Assay Method

Field of the Invention

The present invention relates to drug resistance. In particular, it relates to methods of determining susceptibility to resistance to anti-cancer drugs such as fluoropyrimidines e.g. 5-Fluorouracil (5-FU), antimetabolites e.g. tomudex (TDX) and platinum containing compounds e.g. oxaliplatin.

Introduction

The fluoropyrimidine drug 5-fluorouracil (5-FU) is used in the treatment of many cancers, including gastrointestinal, breast and head and neck cancers. 5-FU is converted intracellularly to fluorodeoxyuridine monophosphate FdUMP, which, together with 5,10-methylene tetrahydrofolate (CH_2THF) forms a stable ternary complex with thymidylate synthase (TS), resulting in enzyme inhibition. TS catalyses the reductive methylation of deoxyuridine monophosphate (dUMP) by CH_2THF to produce deoxythymidine monophosphate (dTMP) and dihydrofolate (Longley et al Nat Rev Cancer, *3*:330-338, 2003). As this reaction provides the sole *de novo* intracellular source of dTMP, which is

essential for DNA replication and repair, TS inhibition results in DNA damage. Non-TS-directed mechanisms of cytotoxicity have also been described for 5-FU, such as misincorporation of fluoronucleotides into DNA and RNA (Longley et al Nat Rev Cancer, *3*:330-338, 2003).

The major limitation to the clinical use of fluoropyrimidines such as 5-FU is acquired or inherent resistance. *In vitro* and *in vivo* studies have demonstrated that increased TS expression correlates with increased resistance to 5-FU (Johnston et al, Cancer Res., *52*: 4306-4312, 1992). Other upstream determinants of 5-FU chemosensitivity include the 5-FU-degrading enzyme dihydropyrimidine dehydrogenase and 5-FU-anabolic enzymes such as orotate phosphoribosyl transferase (Longley et al Nat Rev Cancer, *3*:330-338, 2003).

The use of antimetabolites e.g. tomudex (TDX) and platinum containing compounds e.g. oxaliplatin is similarly limited by resistance.

Given the importance of providing an effective treatment regime to patients quickly, it would be very useful to be able to identify patients who would not be responsive to chemotherapy using particular agents, prior to initiation of therapy.

Summary of the Invention

The present inventors have used DNA microarray technology to investigate changes in the transcriptional profile of the MCF-7 breast cancer cell line following treatment with some chemotherapeutic agents, e.g. 5-FU. The analysis has identified transcriptional target genes that are induced by such agents. The results suggest that the genes identified may be important downstream mediators of tumour cell response to chemotherapy.

Accordingly, in a first aspect of the present invention, there is provided a method of inducing and/or enhancing expression of one or more of the genes of cells of a biological sample,
said genes being the genes encoding one or more of Raf, K-ras, SLAP, phosphoinositide 3-kinase, COP9 homolog (HCOP9), apoptosis specific protein, APO-1 cell surface antigen, FLIP protein, cyclin G, CDC2 , cyclin-dependent protein kinase -2, thymosin β-10, myosin light chain (MLC-2), gelsolin, thymosin β -4, SSAT, spermidine synthase, spermidine aminopropyltransferase, MAT-8 protein, annexin II, annexin IV, FGF receptor 2, transmembrane 4 superfamily protein , chaperonin 10, enoyl-CoA hydratase, nicotinamide nucleotide transhydrogenase, ribosomal protein S28, ribosomal protein L37, L23 mRNA for putative ribosomal protein, and/or ribosomal protein L7;
said method comprising administration of a chemotherapeutic agent to said sample.

The demonstration that chemotherapeutic agents such as fluoropyrimidines such as 5-FU, antimetabolites such as tomudex and platinum containing compounds such as oxaliplatin enhance expression of these genes in cancerous cells suggests that upregulation of these genes may at least partially contribute to the therapeutic effect of the drugs.

Thus, the invention may be used in assays to determine whether or not treatment with a chemotherapeutic agent such as a fluoropyrimidine e.g. 5-Fluorouracil (5-FU), an antimetabolite e.g. tomudex (TDX) and/or a platinum containing compound e.g. oxaliplatin or an analogue thereof may be effective in a particular patient.

Thus, in a second aspect of the present invention, there is provided a method for evaluating in vitro the response of tumour cells from a subject to the presence of a chemotherapeutic agent to predict response of the tumour cells in vivo to treatment with the chemotherapeutic agent which method comprises:
(a) providing an in vitro sample from a subject containing tumour cells;
(b) exposing a portion of said sample of tumour cells to said chemotherapeutic agent;
(c) comparing expression of one or more of the genes encoding Raf, K-ras, SLAP, phosphoinositide 3-kinase, COP9 homolog (HCOP9), apoptosis specific

protein, APO-1 cell surface antigen, FLIP protein, cyclin G, CDC2 , cyclin-dependent protein kinase -2, thymosin β-10, myosin light chain (MLC-2), gelsolin, thymosin β -4, SSAT, spermidine synthase, spermidine aminopropyltransferase, MAT-8 protein, annexin II, annexin IV, FGF receptor 2, transmembrane 4 superfamily protein , chaperonin 10, enoyl-CoA hydratase, nicotinamide nucleotide transhydrogenase, ribosomal protein S28, ribosomal protein L37, and/or ribosomal protein L7 and/or L23 mRNA for putative ribosomal protein with expression of said one or more genes in a control portion of said sample which has not been exposed to said chemotherapeutic agent; wherein enhanced expression in the portion of sample exposed to said chemotherapeutic agent is indicative of sensitivity to said chemotherapeutic agent.

In preferred embodiments of the second aspect of the invention, expression in the sample exposed to said chemotherapeutic agent is considered to be enhanced if the expression is at least 3-fold, preferably at least 4-fold, more preferably at least 5-fold, even more preferably at least 7-fold, yet more preferably at least 10-fold, most preferably at least 12-fold that of the one or more genes in the control portion of said sample which has not been exposed to said chemotherapeutic agent.

In one preferred embodiment of the invention, the chemotherapeutic agent is a fluoropyrimidine. In a particularly preferred embodiment of the invention,

the chemotherapeutic agent is 5-FU or an analogue thereof, most preferably 5-FU.

In another preferred embodiment of the invention, the chemotherapeutic agent is an antimetabolite. A particularly preferred antimetabolite is tomudex or an analogue thereof, most preferably tomudex.

In another preferred embodiment of the invention, the chemotherapeutic agent is a platinum containing compound, for example oxaliplatin, cisplatin or an analogue thereof. A particularly preferred platinum containing compound is oxaliplatin or an analogue thereof, most preferably oxaliplatin.

Furthermore, the invention may also be used to identify novel chemotherapeutic agents.

Accordingly, in a third aspect of the invention, there is provided an assay method for identifying a chemotherapeutic agent for use in the treatment of cancer, said method comprising the steps:
(a) providing a sample of tumour cells;
(b) exposing a portion of said sample to a candidate chemotherapeutic agent;
(c) determining expression of one or more of the genes encoding Raf, K-ras, SLAP, phosphoinositide 3-kinase, COP9 homolog (HCOP9), apoptosis specific protein, APO-1 cell surface antigen, FLIP protein, cyclin G, CDC2 , cyclin-dependent protein kinase -2, thymosin β-10, myosin light chain (MLC-2), gelsolin, thymosin β -4, SSAT, spermidine synthase, spermidine

aminopropyltransferase; MAT-8 protein, annexin II, annexin IV, FGF receptor 2, transmembrane 4 superfamily protein , chaperonin 10, enoyl-CoA hydratase, nicotinamide nucleotide transhydrogenase, ribosomal protein S28, ribosomal protein L37, and/or ribosomal protein L7 and/or L23 mRNA for putative ribosomal protein with expression of said one or more genes in a control portion of said sample which has not been exposed to said candidate chemotherapeutic agent; wherein enhanced expression in the sample exposed to said candidate chemotherapeutic agent compared to expression in the portion of sample not exposed to the candidate chemotherapeutic agent is indicative of chemotherapeutic effect.

In preferred embodiments of the third aspect of the invention, expression in the portion of sample exposed to said candidate chemotherapeutic agent is considered to be enhanced if the expression is at least 3-fold, preferably at least 4-fold, more preferably at least 5-fold, even more preferably at least 7-fold, yet more preferably at least 10-fold, most preferably at least 12-fold that of the one or more genes in the control portion of said sample which has not been exposed to said candidate chemotherapeutic agent.

In one particularly preferred embodiment of the third aspect of the invention, the gene is a gene encoding MAT-8 or, more preferably, chaperonin-10.

Further, the present inventors have also investigated the basal expression levels of the genes identified as being up-regulated in response to chemotherapeutic treatment. Surprisingly, it has been found that basal levels of expression of these genes was dramatically increased in 5-FU resistant cancer cells compared to basal expression levels in 5-FU sensitive cancer cells. This suggests that enhanced expression of one or more of these genes may be used as biomarkers of resistance to 5-FU.

Accordingly, in a fourth aspect of the present invention, there is provided a method to predict response of tumour cells to in vivo treatment with a chemotherapeutic agent, said method comprising the steps:

(a) providing an in vitro sample containing tumour cells from a subject;

(b) determining the basal expression of one or more of the genes encoding Raf, K-ras, SLAP, phosphoinositide 3-kinase, COP9 homolog (HCOP9), apoptosis specific protein, APO-1 cell surface antigen, FLIP protein, cyclin G, CDC2 , cyclin-dependent protein kinase -2, thymosin β-10, myosin light chain (MLC-2), gelsolin, thymosin β -4, SSAT, spermidine synthase, spermidine aminopropyltransferase, MAT-8 protein, annexin II, annexin IV, FGF receptor 2, transmembrane 4 superfamily protein , chaperonin 10, enoyl-CoA hydratase, nicotinamide nucleotide transhydrogenase, ribosomal protein S28, ribosomal protein L37, and/or ribosomal protein L7 and/or L23 mRNA for putative

ribosomal protein, wherein enhanced basal expression of said one or more of the genes compared to the basal expression level of the corresponding gene(s) in one or more control samples is indicative of resistance to a chemotherapeutic agent.

In preferred embodiments of this aspect of the invention the chemotherapeutic agent is a fluoropyrimidine e.g. 5-Fluorouracil (5-FU), an antimetabolite e.g. tomudex (TDX) or a platinum containing compound e.g. oxaliplatin or an analogue thereof

The control samples may be a fluoropyrimidine-sensitive e.g. 5-FU sensitive, platinum containing antineoplastic sensitive e.g. oxaliplatin sensitive and/or antimetabolite sensitive e.g tomudex sensitive cancer cell-line. For example, in a preferred embodiment, the control sample is the H630 5-FU sensitive cancer cell line.

Alternatively, the control samples may be biological samples of cells, tissues or fluid from non-cancerous tissues of human subjects. Human subjects may include cancer patients, subjects free of cancer or both. The basal expression level of the gene(s) in the control sample(s) may be determined in advance to provide control basal expression level value(s) with which to compare the expression level(s) of the in vitro sample.

In preferred embodiments of the invention, the one or more genes are preferably one or more of genes encoding Raf, K-ras, SLAP, phosphoinositide 3-kinase, COP9 homolog (HCOP9), apoptosis specific protein, APO-1 cell surface antigen, FLIP protein, cyclin G, CDC2 , cyclin-dependent protein kinase -2, myosin light chain (MLC-2), gelsolin, thymosin β -4, spermidine synthase, spermidine aminopropyltransferase, annexin IV, FGF receptor 2, transmembrane 4 superfamily protein, enoyl-CoA hydratase, nicotinamide nucleotide transhydrogenase, ribosomal protein S28, ribosomal protein L37, and/or ribosomal protein L7 and/or L23 mRNA for putative ribosomal protein

In another preferred embodiment of the invention, the one or more genes are preferably one or more of genes encoding Raf, K-ras, SLAP, phosphoinositide 3-kinase, COP9 homolog (HCOP9), apoptosis specific protein, APO-1 cell surface antigen, FLIP protein, cyclin G, CDC2 , cyclin-dependent protein kinase -2, myosin light chain (MLC-2), gelsolin, thymosin β -4, spermidine synthase, spermidine aminopropyltransferase, FGF receptor 2, transmembrane 4 superfamily protein, enoyl-CoA hydratase, nicotinamide nucleotide transhydrogenase, ribosomal protein S28, ribosomal protein L37, and/or ribosomal protein L7 and/or L23 mRNA for putative ribosomal protein

In another preferred embodiment of the invention, said one or more genes encodes SSAT, annexin II, thymosin-β-10, MAT-8 or Chaperonin-10.

In a particularly preferred embodiment of the fourth aspect of the invention, the gene is a gene encoding MAT-8.

Preferred features of each aspect of the invention are as for each of the other aspects mutatis mutandis unless the context demands otherwise.

Detailed Description

As described above, the present invention relates to methods of screening samples comprising tumour cells for expression of particular genes in order to determine suitability for treatment using chemotherapeutic agents.

The methods of the invention may involve the determination of expression of one or more of the genes encoding Raf, K-ras, SLAP, phosphoinositide 3-kinase, COP9 homolog (HCOP9), apoptosis specific protein, APO-1 cell surface antigen, FLIP protein, cyclin G, CDC2 , cyclin-dependent protein kinase -2, thymosin β-10, myosin light chain (MLC-2), gelsolin, thymosin β -4, SSAT, spermidine synthase, spermidine aminopropyltransferase, MAT-8 protein, annexin II, annexin IV, FGF receptor 2, transmembrane 4 superfamily protein , chaperonin 10, enoyl-CoA hydratase, nicotinamide nucleotide transhydrogenase,

ribosomal protein S28, ribosomal protein L37, and/or ribosomal protein L7 and/or L23 mRNA for putative ribosomal protein, preferably one or more of the genes encoding SSAT, annexin II, thymosin-β-10, MAT-8 or Chaperonin-10.

The expression of each gene may be measured using any technique known in the art. Either mRNA or protein can be measured as a means of determining up-or down regulation of expression of a gene. Quantitative techniques are preferred. However semi-quantitative or qualitative techniques can also be used. Suitable techniques for measuring gene products include, but are not limited to, SAGE analysis, DNA microarray analysis, Northern blot, Western blot, immunocytochemical analysis, and ELISA.

In the methods of the invention, RNA can be detected using any of the known techniques in the art. Preferably an amplification step is used as the amount of RNA from the sample may be very small. Suitable techniques may include RT-PCR, hybridisation of copy mRNA (cRNA) to an array of nucleic acid probes and Northern Blotting.

For example, when using mRNA detection, the method may be carried out by converting the isolated mRNA to cDNA according to standard methods; treating the converted cDNA with amplification reaction reagents (such as cDNA PCR reaction reagents) in a container along with an appropriate mixture of nucleic acid

primers; reacting the contents of the container to produce amplification products; and analyzing the amplification products to detect the presence of gene expression products of one or more of the genes encoding Raf, K-ras, SLAP, phosphoinositide 3-kinase, COP9 homolog (HCOP9), apoptosis specific protein, APO-1 cell surface antigen, FLIP protein, cyclin G, CDC2 , cyclin-dependent protein kinase -2, thymosin β-10, myosin light chain (MLC-2), gelsolin, thymosin β -4, SSAT, spermidine synthase, spermidine aminopropyltransferase, MAT-8 protein, annexin II, annexin IV, FGF receptor 2, transmembrane 4 superfamily protein , chaperonin 10, enoyl-CoA hydratase, nicotinamide nucleotide transhydrogenase, ribosomal protein S28, ribosomal protein L37, and/or ribosomal protein L7 and/or L23 mRNA for putative ribosomal protein, preferably one or more of the genes encoding SSAT, annexin II, thymosin-β-10, MAT-8 or Chaperonin-10 in the sample. Analysis may be accomplished using Northern Blot analysis to detect the presence of the gene products in the amplification product. Northern Blot analysis is known in the art. The analysis step may be further accomplished by quantitatively detecting the presence of such gene products in the amplification products, and comparing the quantity of product detected against a panel of expected values for known presence or absence in normal and malignant tissue derived using similar primers.

Primers for use in methods of the invention will of course depend on the gene(s), expression of which is

being determined. In preferred embodiments of the invention, one or more of the following primer sets may be used:

SSAT:
Forward, 5'-GCT AAA TTC GTG ATC CGC-3'(SEQ ID NO:1)
Reverse, 5'-CAA TGC TGT GTC CTT CCG-3'(SEQ ID NO: 2)

Annexin II:
Forward, 5'-GGG TGA TCA CTC TAC ACC-3'(SEQ ID NO:3)
Reverse, 5'-CAG TGC TGA TGC AGG TTC-3'(SEQ ID NO:4);

Thymosin β-10:
Forward, 5'-TCG GAA CGA GAC TGC ACG-3'(SEQ ID NO:5)
Reverse, 5'-CTC TTC CTC CAC ATC ACG-3'(SEQ ID NO:6);

MAT-8:
Forward, 5'-GCT CTG ACA TGC AGA AGG-3'(SEQ ID NO:9)
Reverse, 5'-CCT CCA CCC AAT TTC AGC-3'(SEQ ID NO:10)

Chaperonin-10:
Forward, 5'-GTA ATG GCA GGA CAA GCG-3'(SEQ ID NO:11)
Reverse, 5'-GGG CAG CAT GTT GAT GC-3'(SEQ ID NO:12)

In e.g. determining gene expression in carrying out methods of the invention, conventional molecular biological, microbiological and recombinant DNA techniques known in the art may be employed. Details of such techniques are described in, for example, Sambrook, Fritsch and Maniatis, "Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989, and Ausubel et al, Short

Protocols in Molecular Biology, John Wiley and Sons, 1992).

The methods of the invention may be used to determine the suitability for treatment of any suitable cancer with a chemotherapeutic agent e.g. 5-FU, tomudex or oxaliplatin or analogues thereof. For example the methods of the invention may be used to determine the sensitivity or resistance to treatment of cancers including, but not limited to, gastrointestinal, breast, prostate, head and neck cancers.

In particularly preferred embodiments of the invention, the methods of the invention may be used to determine the sensitivity or resistance to treatment of breast cancer.

The nature of the tumour or cancer will determine the nature of the sample which is to be used in the methods of the invention. The sample may be, for example, a sample from a tumour tissue biopsy, bone marrow biopsy or circulating tumour cells in e.g. blood. Alternatively, e.g. where the tumour is a gastrointestinal tumour, tumour cells may be isolated from faeces samples. Other sources of tumour cells may include plasma, serum, cerebrospinal fluid, urine, interstitial fluid, ascites fluid etc.

For example, solid tumours may be collected in complete tissue culture medium with antibiotics.

Cells may be manually teased from the tumour specimen or, where necessary, are enzymatically disaggregated by incubation with collagenase/DNAse and suspended in appropriate media containing, for example, human or animal sera.

In other embodiments, biopsy samples may be isolated and frozen or fixed in fixatives such as formalin. The samples may then be tested for expression levels of genes at a later stage.

As described above, chemotherapeutic agents suitable for use in methods of the invention include fluoropyrimidines e.g. 5-FU, platinum containing compounds e.g oxaliplatin, antimetabolites such as tomudex and analogues thereof. Analogues include biologically active derivatives and substantial equivalents thereof.

"Treatment" or "therapy" includes any regime that can benefit a human or non-human animal. The treatment may be in respect of an existing condition or may be prophylactic (preventative treatment). Treatment may include curative, alleviation or prophylactic effects.

The invention will now be described further in the following non-limiting examples with reference made to the accompanying drawings in which:

Figure 1A illustrates Northern blot analysis of SSAT, annexin II, Thymosin β-10, MAT-8 and

Chaperonin-10 mRNA expression in MCF-7 cells treated for 24, 48 and 72 hours with no drug (-) or 10μM 5-FU (+). 18S rRNA expression was assessed as a loading control.

Figure 1B illustrates Northern blot analysis of SSAT, annexin II, Thymosin β-10, MAT-8 and Chaperonin-10 mRNA expression in MCF-7 cells treated for 72 hours with no drug (Con), 10nM TDX (TDX) or 10μM oxaliplatin (Oxali). 18S rRNA expression was assessed as a loading control.

Figure 2A illustrates Northern blot analysis of SSAT, annexin II, Thymosin β-10, MAT-8 and Chaperonin-10 mRNA expression in p53 wild type M7TS90 cells and p53 null M7TS90-E6 cells treated for 72 hours with no drug (-) or 10μM 5-FU (+). 18S rRNA was assessed as a loading control.

Figure 2B illustrates Western blot analysis of p53 expression in MCF-7 cells treated for 72 hours with no drug (Con), or IC_{60} doses of 5-FU, TDX or oxaliplatin (oxali). GAPDH expression was assessed as a loading control.

Figure 3 illustrates Northern blot analysis of SSAT, annexin II, Thymosin β-10, MAT-8 and Chaperonin-10 mRNA expression in H630 cells treated for 72 h with no drug (Con) or 10μM 5-FU. Basal expression of these genes was also compared in the H630 cell line and the 5-FU resistant H630-R10 daughter line. For

each Northern blot, 18S rRNA expression was used as a loading control.

Examples

Materials and Methods

Tissue culture.

MCF-7 breast cancer and H630 and H630-R10 colon cancer cell lines were maintained in DMEM supplemented with 10% dialyzed fetal calf serum, 1mM sodium pyruvate, 2mM L-glutamine and 50µg/ml penicillin/streptomycin (all from Life Technologies, Paisley, Scotland). M7TS90 cells (Longley et al Cancer Res., *62*: 2644-2649, 2002) were maintained in MCF-7 medium supplemented with 100µg/ml G418 (Life Technologies), 1µg/ml puromycin and 1µg/ml tetracycline (both from Sigma, Poole, Dorset, England). M7TS90-E6 cells (Longley et al Cancer Res., *62*: 2644-2649, 2002) were maintained in M7TS90 medium supplemented with 200µg/ml hygromycin (Life Technologies). All cell lines were grown in 5% CO_2 at 37°C.

Microarray hybridization, detection and scanning RNA was collected from untreated MCF-7 cells (control) or following treatment with 10µM 5-FU for 6, 12, 24 and 48 hours. Ten micrograms of RNA from each timepoint were combined for both the control and 5-FU treated samples. Labeled cDNA probes were prepared from 2µg aliquots of each pooled RNA sample. cDNA synthesized from control cells was labeled with biotin and cDNA synthesized from 5-FU

treated samples was labeled with dinitrophenol (DNP). Labeled probes were purified by ethanol precipitation and membrane-based chemiluminescence analysis was carried out to determine labeling efficiency. The Micromax Human cDNA Array (NEN Lifesciences, Boston, MA) containing 2,400 genes was used in this study. The biotin and DNP labeled cDNA probes were combined and hybridized to the microarray for 16 hours in a humid incubator at 65°C. The microarray was washed in 0.5X SSC and 0.01% SDS for 5 minutes at room temperature with gentle agitation, followed by a 5 minute wash in 0.06X SSC and 0.01% SDS and a 2 minute wash in 0.06X SSC. Hybridized cDNA probes were detected using the Tyramide Signal Amplification (TSA) detection system according to the manufacturer's instructions (NEN Lifesciences). Biotin-labeled cDNA (derived from untreated cells) was visualized using the Cyanine 5 (Cy5) reporter and DNP-labeled cDNA (derived from 5-FU treated cells) was detected using the Cyanine 3 (Cy3) reporter. Scanning of the microarray was performed by NEN Lifesciences (Boston, MA) using a ScanArray confocal laser scanner (GSI Lumonics, Inc). The intensity of each hybridized cDNA was evaluated using ImaGene analysis software (BioDiscovery, Inc) and the Cy3:Cy5 ratio for each gene was calculated.

Northern blot analysis

Northern blots were performed as described previously (Longley et al Cancer Res., *62*: 2644-2649, 2002). DNA probes for Northern blotting were

generated by PCR using cDNA derived from 1µg MCF-7 total RNA as a template. The primer sequences are as follows: SSAT: Forward, 5'-GCT AAA TTC GTG ATC CGC-3'; Reverse, 5'-CAA TGC TGT GTC CTT CCG-3'; Annexin II: Forward, 5'-GGG TGA TCA CTC TAC ACC-3'; Reverse, 5'-CAG TGC TGA TGC AGG TTC-3'; Thymosin β-10: Forward, 5'-TCG GAA CGA GAC TGC ACG-3'; Reverse, 5'-CTC TTC CTC CAC ATC ACG-3'; MAT-8: Forward, 5'-GCT CTG ACA TGC AGA AGG-3'; Reverse, 5'-CCT CCA CCC AAT TTC AGC-3'; Chaperonin-10: Forward, 5'-GTA ATG GCA GGA CAA GCG-3'; Reverse, 5'-GGG CAG CAT GTT GAT GC-3': 18S: Forward 5'-CAG TGA AAC TGC GAA TGG-3'; Reverse 5'-CCA AGA TCC AAC TAC GAG-3'.

Western blot analysis.
Thirty micrograms of protein was resolved by SDS-polyacrylamide gel (12%) as previously described (Longley et al Cancer Res., *62*: 2644-2649, 2002). The gels were electroblotted onto Hybond membranes (Hybond-P, Amersham). Antibody staining was performed with a chemiluminescence detection system (Supersignal, Pierce) using the p53 mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in conjunction with horseradish peroxidase-conjugated sheep anti-mouse secondary antibody. Equal lane loading was assessed using a mouse monoclonal GAPDH antibody (Biogenesis, Poole, UK).

Results

DNA microarray analysis of gene expression following treatment with 5-FU

To identify novel markers of sensitivity or resistance to 5-FU, the inventors carried out cDNA-based microarray analysis following treatment of MCF-7 breast cancer cells with 10μM 5-FU (corresponding to an ~IC_{60} dose at 72hrs). RNA derived from untreated and 5-FU-treated MCF-7 cells was reverse transcribed, labeled and hybridized to a 2,400 gene cDNA microarray. Bound cDNA was detected using Cy3 (5-FU treated) or Cy5 (control) reporter dyes. The expression profile in the treated and untreated populations was compared and expressed as a Cy3:Cy5 ratio. The inventors found that 619 genes (over 25% of genes analyzed) were up-regulated by >3-fold. In contrast, only 16 genes were downregulated by >3-fold, indicating that 5-FU treatment resulted in widespread transcriptional activation. Potential target genes were initially grouped according to their function using the DRAGON database (Database Referencing of Array Genes ONline, http://pevsnerlab.kennedykrieger.org/dragon.htm). The biological functions of the genes identified by the microarray analysis were diverse and include cell cycle regulators, structural, ribosomal, apoptotic and mitochondrial genes, as well as genes involved in signal transduction pathways and polyamine metabolism (Table 1). The manufacturer of the DNA microarray defined changes in gene expression of >3-fold as biologically significant. Our data set was obtained from samples pooled from several timepoints and represents the cumulative increase in gene expression between 6 and 48 hours

after treatment with 5-FU. The inventors selected genes for further investigation on the basis of a cut-off of >6-fold induction and also on the basis of their signal intensities, with intensities of >3,000 considered to be sufficiently high compared to background.

Northern blot analysis of gene expression following treatment with 5-FU

Novel genes that were consistently found to be up-regulated following treatment with 5-FU by Northern blot analysis were SSAT, annexin II, thymosin-β-10, chaperonin-10 and MAT-8 (Fig. 1A). SSAT catalyses the rate-limiting step in the catabolism of the polyamines spermine and spermidine (Hegardt et al, Eur. J. Biochem., *269*: 1033-1039, 2002). SSAT mRNA was induced 15-fold compared to control 48 hours following treatment with 10μM 5-FU, and this induction was maintained at 72 hours (Fig. 1A). Annexin II has been reported to regulate cell proliferation and apoptosis (Chiang et al, Mol. Cell. Biochem., *199*: 139-147, 1999). Induction of annexin II mRNA in response to 5-FU followed a similar pattern to that observed for SSAT with levels ~5-fold higher than control at 72 hours (Fig. 1A). Thymosin- β -10 has also been reported to contribute to the regulation of apoptosis (Hall, A.K. Cell. Mol. Biol. Res., *41*:167-180, 1995). The inventors found that thymosin- β -10 was up-regulated 72 hours after treatment with 5-FU with levels 8-fold above control (Fig. 1A). MAT-8 is a transmembrane protein that regulates chloride ion

transport (Morrison et al. J. Biol. Chem., *270*, 2176-2182, 1995). The inventors found that MAT-8 expression was up-regulated 24 hours after 5-FU treatment and continued to increase throughout the time-course to levels that were 11-fold higher than control by 72 hours (Fig. 1A). Chaperonin-10 is a mitochondrial heat shock protein (Hohfeld and Hartl J. Cell. Biol., *126*: 305-315, 1994). Chaperonin-10 was up-regulated 72 hours post-treatment with 5-FU with levels 4-fold higher than control (Fig. 1A).

Northern blot analysis of target gene expression following treatment with TDX and oxaliplatin.

Recently, specific folate-based inhibitors of TS have been developed, of which Tomudex (TDX) is the first to be approved for clinical use (Hughes et al *In:* AL Jackman (ed.), Antifolate drugs in cancer therapy, pp147-165. Totowa New Jersey: Humana Press, 1999). The platinum-based DNA damaging agent oxaliplatin has demonstrated synergistic activity with TS inhibitors in preclinical studies (Cvitkovic and Bekradda, Semin. Oncol., *26*:647-662, 1999) and is used in the treatment of advanced colorectal cancer (Giacchetti et al, J. Clin. Oncol., *18*:136-147, 2000). The inventors examined the expression of the 5-FU-inducible target genes following treatment of MCF-7 cells with ~IC_{60} doses of TDX (10nM) and oxaliplatin (10μM) for 72 hours (Fig. 1B). SSAT mRNA was up-regulated 15-fold in response to treatment with TDX and 6-fold in response to oxaliplatin (Fig. 1B). Annexin II mRNA was also up-regulated (by ~5-fold) in response to TDX and oxaliplatin. Expression

of thymosin-β-10 mRNA was up-regulated ~5-fold in response to TDX and ~6-fold in response to oxaliplatin (Fig. 1B). MAT-8 mRNA expression was also induced in response to TDX and oxaliplatin by ~8-fold in each case (Fig. 1B). Treatment with TDX caused a moderate 1.5-fold induction of chaperonin-10 and oxaliplatin treatment resulted in ~2.5-fold induction of this gene (Fig. 1B). Thus, the 5-FU target genes identified by the cDNA microarray screen were also found to be induced by TDX and oxaliplatin.

Effect of p53 inactivation on target gene induction. p53 has previously been reported to play an important role in downstream signalling following 5-FU treatment (Longley et al Cancer Res., *62*: 2644-2649, 2002). To determine whether p53 might play a role in 5-FU-mediated target gene up-regulation, the inventors examined the sequences of the 5-FU-inducible genes for regions of homology to putative p53-binding sites using the TRANSFAC database (http://transfac.gbf.de/TRANSFAC, 12). The inventors found that the *SSAT* and *MAT-8* genes each contained 3 putative p53 binding sites with >85% homology and the *annexin II* and *thymosin-β-10* genes each contained 2 sites. The *chaperonin-10* and *hsp60* genes are transcribed from the same promoter and this locus contained 16 putative p53-binding sites. This suggested that p53 might play a role in the regulation of expression of these genes. The inventors therefore compared expression of each of the 5-FU-inducible genes in p53 wild-type (M7TS90)

and p53 null (M7TS90-E6) isogenic cell lines, derived from MCF-7 cells as previously described (Longley et al Cancer Res., *62*: 2644-2649, 2002). In the M7TS90 cell line, SSAT mRNA expression was induced following treatment with 5-FU for 72 hours to a similar extent as in the parental MCF-7 line (~13-fold), while expression in the p53 null M7TS90-E6 cell line was only up-regulated by ~2-fold (Fig. 2A). Induction of annexin II mRNA was also reduced in the p53 null cell line (2-fold with respect to control) compared to the p53 wild-type line (7-fold with respect to control, Fig. 2A). In M7TS90 cells, MAT-8, thymosin- β -10 and chaperonin-10 mRNAs were each induced by 5-FU treatment by between 8-10-fold (Fig. 2A). In contrast, expression of these genes was unaltered by 5-FU treatment in the p53 null M7TS90-E6 cell line (Fig. 2A). These results suggested an important regulatory role for p53 in up-regulating each of these target genes, therefore, the inventors also examined the effect of 5-FU, TDX and oxaliplatin on p53 protein expression. MCF-7 cells were exposed to ~IC_{60} doses of each agent for 48 hours (Fig. 2B). p53 protein levels were up-regulated following exposure to 10μM 5-FU (7-fold), TDX (3-fold) and oxaliplatin (8-fold, Fig. 2B). Collectively these results suggested a key transcriptional regulatory role for p53 in the response to 5-FU, TDX and oxaliplatin in this cell line.

Expression of target genes in the 5-FU resistant H630-R10 cell line

The inventors next examined the expression of the validated target genes in H630 colon cancer cells following exposure to 5-FU (Fig 3). The inventors discovered that expression of SSAT and MAT-8 mRNA in H630 cells was induced by ~5-6-fold following treatment with 10μM 5-FU. Chaperonin-10 mRNA expression was also up-regulated by ~3-fold in response to 5-FU, however the expression of annexin II and thymosin-β-10 mRNA was only marginally up-regulated (by ~2-fold) following exposure to 10μM 5-FU (Fig. 3). The inventors also compared basal expression of the 5-FU-inducible genes in the H630 colorectal cancer cell line and a 5-FU resistant daughter line, H630-R10. The inventors found that expression of MAT-8 mRNA was dramatically increased in the 5-FU resistant H630-R10 cell line compared to the parental H630 cell line (by ~10-fold, Fig. 3B). Expression of SSAT, annexin II and thymosin-β-10 mRNAs were also elevated in the resistant cell line (by ~2-fold in each case), while chaperonin-10 expression levels were ~3-fold higher in H630-R10 cells compared to H630 cells (Fig. 3B). Thus, the development of 5-FU resistance in H630-R10 cells correlated with increased basal expression of each of the target genes.

Discussion

In the present study, the inventors have used the assessment of gene-expression profiles by cDNA microarray following treatment with chemotherapeutic

agents to identify genes that are up-regulated following treatment with 5-FU in MCF-7 breast cancer cells. Of 2,400 genes analyzed, the inventors found that 619 genes (over 25%) were up-regulated by >3-fold, highlighting the widespread up-regulation of gene expression caused by 5-FU treatment. To initially characterize the genes that were transcriptionally activated by 5-FU, the inventors grouped them according to function using the DRAGON database (Table 1). The inventors identified several families of up-regulated genes, including genes encoding structural, mitochondrial, ribosomal and cell surface proteins, and genes involved in the regulation of cell cycle, apoptosis and polyamine metabolism. The expression of a number of genes implicated in signal transduction pathways was also up-regulated in response to 5-FU.

The manufacturer of the cDNA microarray recommended that >3-fold induction could be considered biologically significant. However, our data set was generated using RNA samples collected at several timepoints following 5-FU treatment. As these samples were pooled prior to analysis, our data set represents the cumulative changes in gene expression between 6 and 48 hours post-drug treatment. The inventors used a cut-off of >6-fold induction when selecting genes for further validation and further characterization. The inventors also used a signal intensity cut-off of >3,000 to ensure identification of genes with signals of sufficient intensity to minimize the effects of background noise. The

inventors demonstrated that spermine/spermidine acetyl transferase (SSAT), annexin II, thymosin-β-10, MAT-8 and chaperonin-10 were consistently up-regulated following treatment with an IC_{60} dose of 5-FU in MCF-7 cells. SSAT causes a reduction in intracellular polyamine levels, which is associated with the induction of apoptosis (Hegardt et al, Eur. J. Biochem., *269*: 1033-1039, 2002). Annexin II is a member of the annexin family of genes and has been implicated in numerous roles including the regulation of DNA synthesis, cell proliferation and apoptosis (Chiang et al, Mol. Cell. Biochem., *199*: 139-147, 1999). The G-actin binding protein thymosin-β-10 is a member of the β-thymosin family of proteins (Yu et al, J. Biol. Chem., *268*: 502-509, 1993) and plays a role in the regulation of apoptosis (Hall, A.K. Cell. Mol. Biol. Res., *41*:167-180, 1995). MAT-8 is a member of the FXYD family of proteins (Sweadner and Rael, Genomics, *68*: 41-56, 2000) that regulates chloride ion transport across the cell membrane (Morrison et al. J. Biol. Chem., *270*, 2176-2182, 1995). The heat shock protein chaperonin-10 (hsp10) binds hsp60 to regulate folding of mitochondrial proteins (Hohfeld and Hartl J. Cell. Biol., *126*: 305-315, 1994). To our knowledge, none of these genes have been previously identified as 5-FU-inducible target genes.

The inventors found that $\sim IC_{60}$ doses of the TS-targeted antifolate TDX and the DNA damaging agent oxaliplatin also caused up-regulation of each of the target genes. Each of these genes was found to

contain potential p53-responsive elements. Importantly, inactivation of p53 in an MCF-7-derived cell line (M7TS90-E6) resulted in significantly reduced levels of 5-FU-mediated induction of SSAT and annexin II mRNA, while expression of thymosin-β-10, MAT-8 and chaperonin-10 was not induced in the p53 null setting. These results suggest that p53 may play a role in regulating expression of the target genes in response to 5-FU. In addition, p53 protein was induced in MCF-7 cells treated with ~IC_{60} doses of 5-FU, TDX and oxaliplatin. Thus, these agents induced target gene expression and also caused up-regulation of p53, providing further evidence for the involvement of p53 in regulating these genes.

The inventors also examined expression of the validated target genes in the H630 colorectal cancer cell line and the paired 5-FU resistant daughter cell line, H630-R10 (Johnston et al, Cancer Res., *52*: 4306-4312, 1992). TS is overexpressed in the H630-R10 cell line by 33-fold compared to the parental line. The inventors found that expression of all five target genes was up-regulated in response to 5-FU in the H630 parent cell line. Interestingly, the inventors also found that basal expression of all five target genes, in particular MAT-8, was higher in the 5-FU-resistant H630-R10 daughter cell line. Without being limited to any one theory, this may arise due to the dysregulation of target gene expression in the 5-FU resistant cell line, as elevated basal expression of these genes

was not associated with increased cell cycle arrest or apoptosis. Thus, H630-R10 cells may tolerate higher basal levels of the target genes, suggesting they may be potential biomarkers of resistance.

A key concern with the use of cDNA microarray analysis in relation to cancer therapy is that the evaluation of a large number of genes may identify such a sizeable number of potential target genes that it would be unfeasible to try to confirm the involvement of each of these genes in resistance/response to therapy. Nonetheless, the present study has shown that microarray analysis is a powerful technology for the identification of novel genes associated with response or resistance to chemotherapeutic agents.

In conclusion, using DNA microarray technology, the inventors have identified thirty 5-FU-inducible transcriptional targets (see Table 1). These include SSAT, annexin II, MAT-8, thymosin β-10 and chaperonin-10. These genes were also up-regulated by TDX and oxaliplatin. Each of these genes contains putative p53-response elements and 5-FU-mediated induction of these genes was significantly reduced in a p53 null MCF-7 daughter line, suggesting a role for p53 in their regulation. Finally, basal expression of these genes (in particular MAT-8) was higher in a 5-FU resistant cell line, suggesting that these genes may be potential biomarkers of 5-FU resistance. These results demonstrate the potential of DNA microarrays to identify novel genes involved

in mediating the response of tumour cells to chemotherapy.

All documents referred to in this specification are herein incorporated by reference. Various modifications and variations to the described embodiments of the inventions will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes of carrying out the invention which are obvious to those skilled in the art are intended to be covered by the present invention.

Table 1

FAMILY	EXAMPLES	FOLD INDUCTION	SIGNAL INTENSITY
Signal transduction	Raf	3.9	8686
	K-ras	4.8	9662
	SLAP (SRC-like adaptor protein)	5.0	5391
	Phosphoinositide 3-kinase	3.2	918
Apoptosis	COP9 homolog (HCOP9)	8.6	1587
	Apoptosis specific protein	4.6	1625
	APO-1 cell surface antigen	4.2	4453
	FLIP protein	3.7	5793
Cell cycle	Cyclin G	8.5	13789
	CDC2	3.1	1779
	Cyclin-dependent protein kinase -2	5.9	3416
Structural	Thymosin β-10	8.5	27041
	Myosin light chain (MLC-2)	3.2	397
	Gelsolin	7.3	18482
	Thymosin β-4	4.3	46355
Polyamine metabolism	SSAT	13.0	3662
	Spermidine synthase	3.7	3874
	Spermidine aminopropyltransferase	5.0	6633
Cell surface	MAT-8 protein	10.1	6522
	Annexin II	12.3	24463
	Annexin IV	9.3	4101
	FGF receptor 2	4.9	684
	Transmembrane 4 superfamily protein	3.2	491
Mitochondrial	Chaperonin 10	11.6	8478
	Enoyl-CoA hydratase	3.4	2512
	Nicotinamide nucleotide transhydrogenase	4.7	1508
Ribosomal proteins	Ribosomal protein S28	10.9	24039
	Ribosomal protein L37	3.0	723
	L23 mRNA for putative ribosomal protein	4.6	12662
	Ribosomal protein L7	5.5	1724